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Hepatitis C (HCV) Genotype and Viral Titer Distribution Among Argentinean Hemophilic Patients in the Presence or Absence of Human Immunodeficiency Virus (HIV) Co-Infection

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Hepatits C (HCV) infection is frequent among hemophilic patients treated with non-inactivated factor-concentrates. Both HCV genotype and viral load have been suggested to be important prognostic markers of disease progression and treatment outcome. In addition, co-infection with the human immunodeficiency virus (HIV) has been associated with increased level of HCV replication and higher risk of developing liver failure. Thus, HCV genotype, viral load, and HIV co-infection are important factors in HCV infection.

Using restriction fragment length polymorphism analysis (RFLP) and the branched-DNA (bDNA) assay, we retrospectively investigated the HCV genotypes and viral loads present in 59 Argentinean hemophiliacs, in the presence or absence of HIV infection. HCV genotype 1 was the predominant viral variant detected among HIV-negative (HIV^-) (76%) and HIV-positive (HIV^+) (82.5%) patients, followed by genotypes 3 (10.4%), 2 (2%) and a small proportion of multiply co-infected patients including genotypes 4 and 5 (6.25%). HIV^+ patients had higher plasma HCV RNA levels than HIV^- patients ($88.4 \pm 16.5 \times 10^5$ Eq/ml vs. $24.7 \pm 5.8 \times 10^5$ Eq/ml) ($P < 0.001$); however, no correlation between HCV replication and level of immune suppression, evaluated by $CD4^+$ T-cell measurement, was observed among HIV^+ patients ($r = 0.017$). Abnormal and higher ALT levels were more frequently detected among HIV^+ (93%; 123.6 ± 15.7 U/liter) than HIV^- (41%; 70.2 ± 24.2 U/liter) patients ($P < 0.001$; $P < 0.05$). Although we were able to confirm previous reports suggesting the existence of increased HCV replication in HIV/HCV co-infected hemophiliacs, our data did not support the conclusion that HIV-induced immune suppression is directly responsible for this phenomena. It is possible that other factors induced by HIV are responsible for the increased levels in HCV replication observed. ***J. Med. Virol. 52:219–225, 1997.***

KEY WORDS: hepatitis C genotype; hemophiliacs; viral load

INTRODUCTION

Infection with the Hepatitis C (HCV) virus, the causative agent of the majority of cases of non-A, non-B hepatitis, is found in the vast majority of hemophilic patients treated with non-inactivated clotting factor concentrates [Brettler et al., 1990; Makris et al., 1990]. The introduction of inactivation procedures and blood donor screening for antibodies against HCV, has dramatically improved the safety of pooled plasma products [Guo et al., 1995]. As a consequence, HCV infection has become a rare event among hemophilic patients in recent years. HCV infection in the Argentinean hemophilic population reflects these trends. In previous studies, we determined the HCV seroprevalence among patients born before and after the introduction of inactivated factor concentrates [Picchio et al., 1994]. Our results indicated that 89% of hemophiliacs receiving non-inactivated factor concentrated have antibodies against HCV, while a near absence of new

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infections was detected among patients born after 1990. Over 70% of HCV seropositive (HCV^+) patients had detectable levels of viremia when analyzed by nested PCR.

A large proportion of hemophilic patients transfused with untreated clotting factor concentrates between 1978 and 1984 also became infected with the human immunodeficiency virus (HIV) [Eyster et al., 1993]. Thus, co-infection with HIV and HCV is a frequent finding among hemophiliacs. Previous reports have shown that liver failure occurs more frequently in HCV^+ hemophiliacs who are also HIV-seropositive (HIV^+) than in those who are HIV-seronegative (HIV^-) [Telfer et al., 1995; Eyster et al., 1994]. Moreover, Eyster et al. [1994] found that HCV RNA levels were higher in HIV^+ than HIV^- hemophiliacs and that HCV RNA levels inversely correlated with $CD4^+$ T cell levels. These findings suggested that HIV-induced immune deficiency may allow increased HCV replication. However, although several recent reports have been able to confirm the existence of higher HCV RNA levels among HIV^+/HCV^+ hemophiliacs and IVDU [Sherman et al., 1993; Chambost et al., 1995; Cribier et al., 1995], none have demonstrated any correlation between HCV plasma viremia and $CD4^+$ T cell levels.

The importance of HCV RNA levels may also be influenced by the HCV genotype. The HCV genome exhibits a considerable degree of polymorphism. Variants can be classified into six genotypes, each with a variable number of more closely related subtypes [Simmonds et al., 1993]. It is now well established that different genotypes can be identified in particular geographic regions [McOmish et al., 1994]. Differences in clinical features such as disease severity or responsiveness to interferon-α (IFN-α) treatment have been described for genotypes 1 and 2. Infection with HCV type 1, especially 1b [Yoshioka et al., 1992; McOmish et al., 1994], has been associated with faster progression to cirrhosis and low response rates to treatment with IFN-α, while infection with HCV type 2 has been associated with less severe disease [Kobayashi et al., 1996]. It has been suggested that pre-existing HCV RNA levels may influence the likelihood of achieving sustained responses after IFN-α treatment [Lau et al., 1993], and that this variable may be more important than virus genotype in predicting response to treatment [Kobayashi et al., 1993].

Therefore, it may be important to assess HCV genotype and plasma viral RNA levels as well as co-infection with HIV in planning treatment for HCV infection. In this retrospective study, we investigated these parameters in Argentinean hemophilic patients.

PATIENTS, MATERIALS, AND METHODS

A total of 59 samples were collected from 59 HCV^+ hemophilic patients, 29 HIV^- (mean age 20.5 years, range 6–47) and 30 HIV^+ (mean age 26.5 years, range 11–50) between 1993 and 1994. The presence of anti-HCV antibodies was determined with a second generation EIA test (Ortho Diagnostics, Raritan, NJ) according to the manufacturer's recommendations. Samples were further analyzed with a second generation recombinant immunoblot assay (RIBA 2.0; Chiron Corporation, Emeryville, CA). All patients included in the study had detectable plasma HCV RNA determined by a nested PCR test performed as previously described [Chan et al., 1992].

Viral Load Determination

HCV viral load was determined using the branched-DNA (bDNA) hybridization system version 1.0 (Chiron Corporation, Emmeryville, CA) according to the manufacturer's specifications. The cut-off value of this assay is 3.5×10^5 Eq/ml. It has recently been described that the HCV bDNA assay 1.0 version may underestimate viral load levels derived from genotypes 2 and 3 [Collins et al., 1995]. To overcome that problem, we reanalyzed with the HCV bDNA assay 2.0 version all samples presenting genotypes 2 or 3 ($n = 8$).This assay has a limit of detection of 2.0×10^5 Eq/ml and detects genotypes 1, 2, and 3 with similar efficiency [Detmer et al., 1996]. HIV viral load was also determined using the bDNA method (version 2.0). However, since the volume of sample used for HIV RNA determination was only 50 μl instead of the regular 1 ml used in this assay, the cut-off value of this assay was established as 1×10^4 Eq/ml.

HCV Genotyping

Due to the retrospective nature of this study, the available volume of each sample was not sufficient to perform the genotype determination in all 59 samples analyzed for viral load. Accordingly, 23 patients belonging to the HIV^+ group and 25 from the HIV^- could be analyzed. HCV genotyping was carried out using two different methodologies. All samples were initially genotyped using Restriction Fragment Length Polymorphism (RFLP) analysis of PCR-amplified 5′-noncoding region as previously described [Davidson et al., 1995]. Briefly, 20 μl of PCR products were digested with the following restriction enzymes: a) *Hae*III + *Rsa*I, b) *Mva*I + *Hin*fI, c) *Bst*UI, and d)*Scr*FI. Restriction fragments were separated on a 6% acrylamide gel and stained with ethidium bromide for visualization. This method allows to distinguish the following genotypes and subtypes: 1a, 1b, 2a, 2b, 3a, 3b, 4, 5, and 6. In addition, a total of 11 samples including those with multiple infections were genotyped using the Inno-LIPA (Innogenetics, Zwijnaarde, Belgium) assay to further confirm the RFLP results.

Statistical Analysis

Values were expressed as mean ± S.E. The means were compared by the *t*-test and the x^2 or Fisher's exact test where appropriate. A level of $P < 0.05$ was accepted as being statistically significant.

RESULTS

HCV viremia was detectable and quantifiable by the bDNA assay in 90.0% of HIV^+ vs. 62% in HIV^- patients

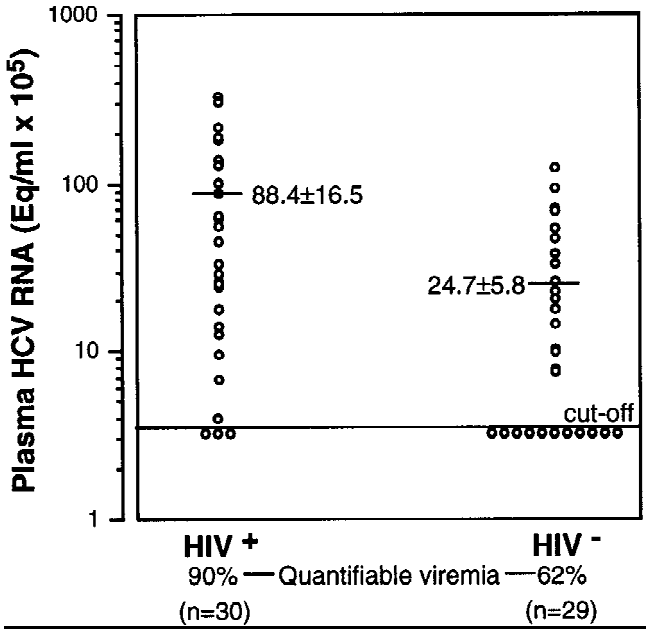

Fig. 1. PlasmaHCV-RNA levels in HIV+ and HIV− hemophiliacs analyzed by b-DNA version 1.0 (genotype 1 and non-genotyped samples) and version 2.0 (genotpe 2 and 3 samples). To simplify, only the cut-off value corresponding to the 1.0 version assay is shown and all samples below this level were plotted together. Of all samples analyzed with the b-DNA assay version 2.0 (n = 8), only one fall between the cut-off levels of both assays, and two were below the 2.0 Eq/ml × 10^5 cut-off level. Samples below the cut-off level were included in the calculation of the mean for each group and were assigned a value similar to the cut-off level depending on the version of bDNA assay used. Horizontal bars indicates the mean HCV RNA level ± standard error. The median HCV RNA level for the HIV+ group was 50.5 Eq/ml × 10^5 and 10.1 Eq/ml × 10^5 for the HIV− group.

($P < 0.05$). A significant difference in the mean HCV viral load was noted between HIV+ (88.4 ± 16.5 × 10^5 Eq/ml) and HIV− patients (24.7 ± 5.8 × 10^5 Eq/ml) ($P < 0.001$) (Fig. 1). In addition, indeterminate RIBA 2.0 results were more frequently observed among HIV+ (30%) than HIV− (6.8%) patients; however, this difference did not achieve statistical significance. There was a tendency to observe more frequently indeterminate RIBA 2.0 results among HIV+ patients with low CD4+ T cell levels and AIDS (clinical stage C) (62.5%) than in patients in clinical stages A and B (18%) ($P > 0.1$) (Table I).

The HCV genotype and subtype distribution from both groups of patients is shown in Figure 2A. HCV genotype 1 was the viral variant most frequently detected in both HIV+ (82.5%) and HIV− (76.0%) hemophiliacs. One HIV+ patient infected with genotype type 1 could not be subtyped. Additional genotypes detected among HIV+ patients were 2b (4.3%), 3a (4.3%), 3b (4.3%), and a single multiple co-infected patient. Among HIV− individuals, genotype 3a was detected in 16% of the cases while 2 (8.0%) patients presented infection with multiple genotypes including genotypes 4 and 5. Interestingly, the subtype distribution among genotype 1 infected patients was different between the groups. Although an almost equal frequency of infection with subtype 1a (40.0%) and 1b (36.0%) was detected among HIV− patients, and increased detection of genotype 1a (56.5%) over 1b (21.7%) ($P < 0.001$) was observed among HIV+ patients (Fig. 2A). The HCV genotype specific mean viral load levels are shown in Figure 2B. No significant intra-group differences in viral load levels were detected between patients infected with genotypes 1a or 1b ($P > 0.1$) (Fig. 2B).

We further investigated whether HCV plasma viral load was associated with two different disease stage-defining parameters of HIV infection among HIV+ hemophiliacs. No correlation between CD4+ lymphocyte levels and HCV plasma viral load was observed (r = 0.017) (Fig. 3A). Furthermore, no significant difference in the mean HCV viral load was observed between patients having detectable vs. non-detectable HIV plasma RNA ($P > 0.5$) (Fig. 3B). Only 12 patients had both detectable HIV and HCV plasma RNA. No clear correlation between these variables was observed in this group of patients (r = 0.241).

Abnormal ALT (normal range 5–55 IU/l) levels were detected among 41% of HIV− hemophilic patients but were not significantly associated with level of HCV viremia or infecting genotype (data not shown). Among HIV+ patients, 93% showed evidence of ALT elevation although no clear association with HCV viremia or clinical stage could be established (Table I and data not shown); however, HIV+ patients infected with genotype 1b had higher mean ALT levels than HIV+ patients infected with genotype 1a (179 ± 38 vs. 102 ± 14 $P = 0.026$) Comparing both groups of patients, mean ALT levels were higher in HIV+ (123.6 ± 15.7 U/liter) than in HIV− patients (70.2 ± 24.2 U/liter) ($P < 0.05$).

DISCUSSION

Our results provide the first report of HCV genotype and viral RNA level distribution among Argentinean HIV+ and HIV− hemophilic patients. In a previous report , Oubiña et al. [1995] investigated HCV genotypes in 10 Argentinean HIV− pediatric hemophilic patients and did not provide viral load levels. Several reports have extensively addressed the issue of genotype distribution among different populations of hemophilic patients worldwide [Telfer et al., 1995; Tagariello et al., 1995; Chambost et al., 1995; Isobe et al., 1995; Sheng et al., 1995; Preston et al., 1995]. In agreement with most of them, we have observed a predominance of infection with HCV genotype 1, followed by genotypes 3 and 2. It is likely that the genotype distribution in infected hemophiliacs is a reflection of the viral variants present in the geographic regions where the therapeutic materials are manufactured. Our patients have been treated with commercial factors concentrates from European and North American origin since 1980. HCV genotype 1, 2, and 3 have been frequently reported in United States and Europe [McOmish et al., 1994]. As previously described [Tagariello et al., 1995; Preston et al., 1995; Sheng et al., 1995], we also detected a low frequency of multiple infected patients. Unexpectedly, one patient was co-infected with HCV types 4 and 5 and we have recently identified another co-infected pa-

TABLE I. Summary of Results Obtained From HIV+ Hemophiliacs*

Patient	ALT (IU/liter)	HCV-RNA (Eq/ml × 10^5)	HCV Genotype	RIBA-II	Clinical[b] Stage	Relative CD4 Level (%)	Absolute CD4 Level (cells/μl)	HIV-RNA (Keq/ml)
JA51[a]	290	188	1b	P	A	32	480	<10
IC51[a]	84	25.21	1a	P	A	25	600	<10
LC64	100	127.8	1a	P	A	23	210	<10
SN84	110	55.84	1a	P	B	16	260	13.68
AN67	100	14.2	ND	P	A	25	510	11.82
CP66	110	136	1b	P	B	19	270	73.8
ER83	226	63.51	1a,2a,3b	P	A	23	350	<10
MT82	68	17.9	ND	P	B	10	190	22.7
RMA32	96	39.9	3b	I	B	20	223	<10
JLP44[a]	90	133	1a	P	A	26	570	<10
FS87[a]	242	212.3	1b	P	A	33	740	<10
MV80	90	100	ND	I	C	7	50	40.4
CM43	72	24.63	1a	I	C	11	145	<10
CH39	94	300	1b	P	B	18	227	38.26
RMA34	112	9.6	1a	I	B	19	206	<10
JM74[a]	160	26.11	1b	P	A	34	560	<10
JN31	84	89.12	1a	P	C	14	477	<10
JP68	238	323.8	1a	P	C	15	200	33.82
AB28	60	126.9	1a	I	C	3	10	69.5
CD65	172	6.8	1	P	C	1	10	146.5
DM50	112	45.3	1a	I	C	3	20	142.1
BC92	140	132	1a	P	A	13	210	29.9
SG91	56	3.9	3a	P	A	22	570	<10
PLU95	38	213	2b	I	A	21	400	<10
MM63	86	182	ND	P	A	22	450	<10
CS93[a]	90	33.8	1a	I	A	30	830	<10
LC97	84	<3.5	ND	P	B	19	330	<10
MB60	32	<3.5	1a	P	B	26	410	20.86
DV89[a]	340	<3.5	ND	P	A	34	878	<10
CB42	114	12.47	ND	I	C	2	30	36

*ND = Not determined, P = positive, I = indeterminate.
[a]Long-term survivor.
[b]Centers for Disease Control and Prevention 1993 classification.

tient with genotype 4 infection (unpublished observation). Both of these genotypes, have been shown to be restricted in their geographic distribution to the Middle East and Zaire, [Simmonds et al., 1993] and South Africa [Cha et al., 1992] respectively. We are unaware of reports describing these genotypes in Argentinean blood donors. Other reports [Preston et al., 1995] and our own results suggest that it is most likely that these patients acquired these infections from commercial clotting factors.

Our study population was characterized by a higher proportion of genotype 1 infections both among HIV+ (82.5%) and HIV− (76%) hemophiliacs (Fig. 2A) than previously described [Telfer et al., 1995; Tagariello et al., 1995; Chambost et al., 1995; Isobe et al., 1995; Sheng et al., 1995; Preston et al., 1995]. This observation has important therapeutic implications in view that HCV type 1 infections have been associated with decreased responsiveness to IFN-α treatment and increased risk of disease progression [Telfer et al., 1995; Devereux et al., 1995]. We also detected a different subtype frequency distribution between HIV+ and HIV− patients (Fig. 2A). Assuming that HCV subtype distribution was initially similar in HIV+ and HIV− hemophiliacs, it could be speculated that this difference reflects a decreased survival of HIV+ patients co-infected with genotype 1b. As a group, HIV+ patients co-infected with genotype 1b presented the highest mean HCV viral load observed in our study (Fig. 2B). In addition, the mean ALT level in this subset of patients was significantly higher than the one observed in HIV+ patients infected with genotype 1a. This observation is in agreement with previous reports suggesting the occurrence of a more severe liver disease in patients infected with genotype 1b [Yoshioka et al., 1992; McOmish et al., 1994]. Unfortunately, we do not possess archival samples around the time of HIV seroconversion from co-infected hemophiliacs to determine the initial HCV subtype distribution.

Within the HIV+ or HIV− groups, we did not detect any significant differences in the mean viral load levels between subtype 1a and 1b infected patients, in agreement with previous reports [Smith et al., 1996] (Fig. 2B). The low representation of HCV genotypes 2 and 3 in our study population, did not allow us to draw consistent conclusions on the viral loads levels observed in patients infected with these genotypes. However, it is worth mentioning that only 1/5 patients infected with genotype 3 had HCV RNA levels over 4×10^5 Eq/ml and 2/5 had levels below 2×10^5 Eq/ml when analyzed with the bDNA assay version 2.0. The low viral load detected in our patients is in agreement with the higher frequency of complete clinical responders to IFN-α treatment seen by Devereux et al. [1995] in type 3 infected hemophiliacs. These results should be taken with caution since McOmish et al. [1993] have reported

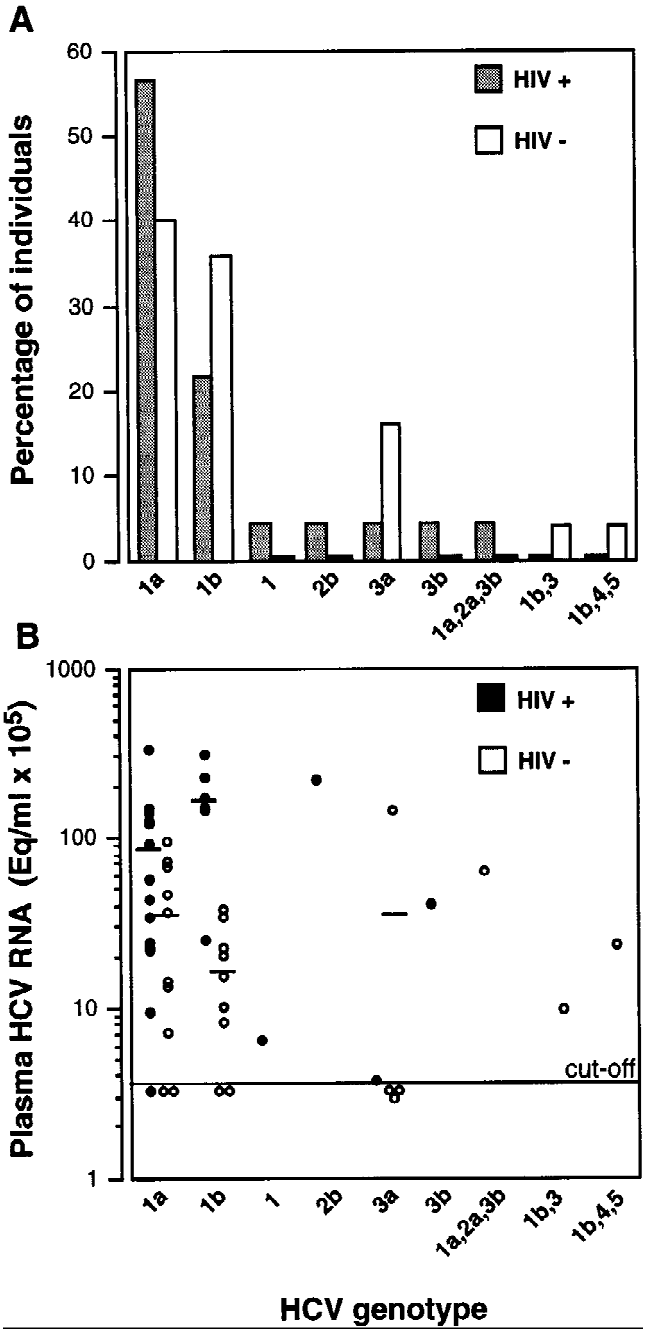

Fig. 2. HCV genotype frequency distribution and mean plasma HCV RNA level according to infecting genotype in HIV⁺ and HIV⁻ hemophilic patients. **A**: HCV genotype distribution among 23 HIV⁺ and 25 HIV⁻ Argentinean hemophilic patients. The × axis only represents the genotypes (single infection or co-infections) observed in our study population. **B**: Mean plasma HCV RNA levels according to genotype. Samples were analyzed with both versions of the bDNA assay as described in Figure 2. The mean HCV RNA level ± standard error for each genotype is indicated with an horizontal bar (HIV⁺ 1a = 86.9×10^5 Eq/ml ± 23.9×10^5; HIV⁻ 1a = 36.4×10^5 Eq/ml ± 10.3×10^5; HIV⁺ 1b = 172.4×10^5 Eq/ml ± 45.1×10^5; HIV⁻ 1b = 17.6×10^5 Eq/ml ± 4.2×10^5; HIV⁻ 3a = 35.1×10^5 Eq/ml ± 33.1×10^5). The median HCV RNA level for each genotype was: HIV⁺ 1a = 55.8×10^5 Eq/ml; HIV⁻ 1a = 26.5×10^5 Eq/ml; HIV⁺ 1b = 188×10^5 Eq/ml; HIV⁻ 1b = 17.7×10^5 Eq/ml; HIV⁻ 3a = 2×10^5 Eq/ml.

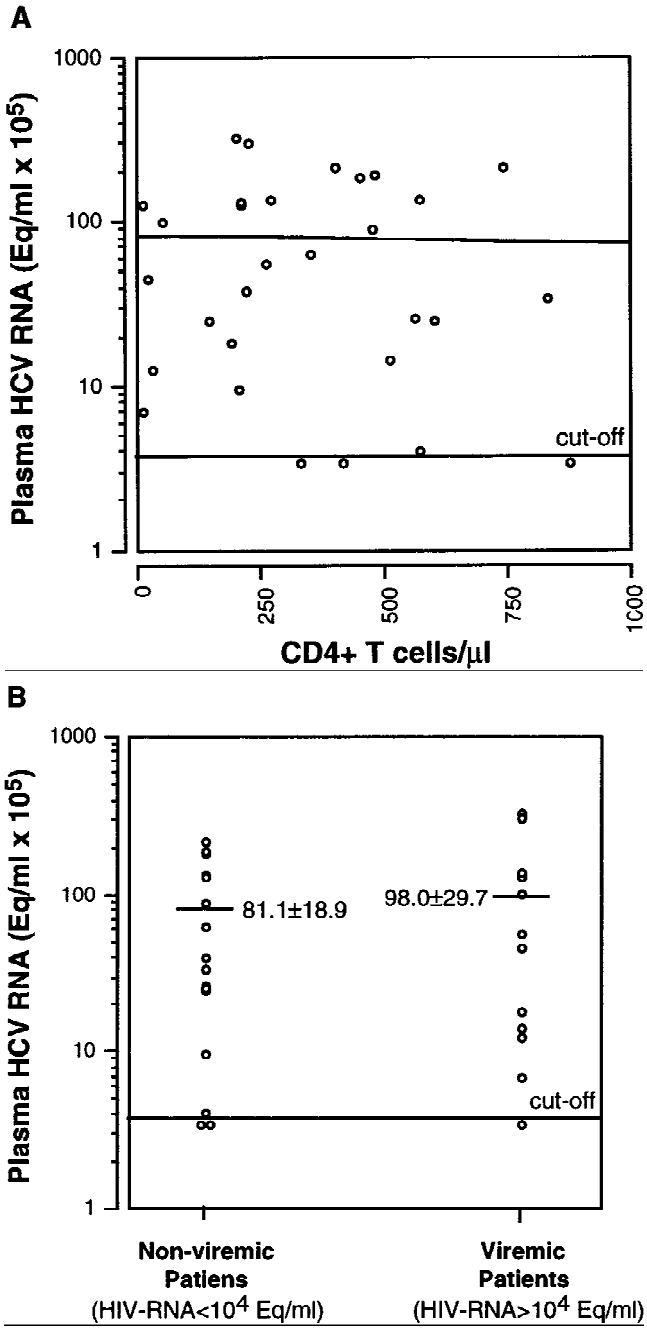

Fig. 3. Correlation between plasma HCV RNA levels and two disease-stage defining parameters of HIV infection among HIV⁺ hemophiliacs. The same considerations mentioned in previous figures regarding HCV RNA determination with the bDNA assay apply. **A**: Correlation between CD4⁺ T cell levels determined by flow cytometry and plasma HCV RNA levels. No correlation was observed between these two parameters ($r = 0.017$). **B**: HIV⁺ patients were divided into HIV viremic or non-viremic according to the presence of plasma HIV RNA levels above or below 10^4 Eq/ml respectively. The mean plasma HCV viral load was calculated for each group and compared. Horizontal bars indicate the mean HCV RNA level ± standard error. No significant difference was observed ($P > 0.05$). The median HCV RNA level for the HIVviremic group was 39.9 Eq/ml × 10^5 and 55.8 Eq/ml × 10^5 for the HIV non-viremic group.

that ALT levels were significantly higher in blood donors infected with HCV type 3.

We find that HCV viral RNA levels are increased in HIV-infected patients (Figs. 1 and 2B), in agreement with the results of Chambost et al. [1995] and Cribier et al. [1995]. This finding was associated with an increased detection of abnormal (93% vs. 41%, $P < 0.001$), and higher (123.6 ± 15.7 U/liter vs. 70.2 ± 24.2 U/liter, $P < 0.05$) ALT levels among HIV$^+$ patients. The detection of a higher proportion of HIV$^+$ patients presenting evidence of liver dysfunction evaluated by serum ALT levels, in particular those infected with genotype 1b (as mentioned above), is in agreement with previous reports suggesting an increased risk of liver failure in co-infected patients [Eyster et al., 1994; Telfer et al., 1994]. On the other hand, the absence of a linear correlation between HCV RNA and ALT levels suggests that factors other than increased HCV replication may be the cause of the increased severity of the liver disease reported to occur in these patients.

Although there was a difference in the mean age between HIV$^+$ and HIV$^-$ patients, it is unlikely that this factor could have accounted for the differences observed, as no overall correlation between patient's age and HCV RNA level was found by us (data not shown) and others [Telfer et al. 1995]. Moreover, in agreement with Cribier et al. [1995], we did not find an association between plasma HCV viral load and degree of immune supression evaluated either by CD4$^+$ T cell, or plasma HIV RNA measurement (Fig. 3A, Table I, and data not shown). In addition, no association between HCV viral load and clinical stage of HIV infection could be found (Table I). This observation was extended to two HCV seropositive hemophilic AIDS patients not included in this study in whom we could not detect viremia by nested PCR (data not shown). Even more striking, was the finding that HIV$^+$ patients JA51, JLP44, and FS87 (Table I), who presented some of the highest HCV viral load levels observed in our study (Table I), are considered long-term survivors since they have been infected with HIV for more that 12 years and yet they maintain normal CD4$^+$ lymphocyte numbers. Attempts to isolate HIV from these patients have yielded negative results over a 2 year period with exception of patient FS87 [manuscript in preparation]. These examples add additional support for the conclusion that the level of immune supression does not necessarily explain the observed differences in HCV replication in HIV$^+$ patients. Thus, understanding what role does the immune system play in controlling HCV replication during chronic infection becomes a critical question. There is certainly evidence that supports the participation of an immune-mediated control of viral replication during chronic HCV infection. Introduction of immunosupressive regimens in cases of HCV$^+$ auto-immune hepatitis have led to an increase in plasma HCV RNA levels. Recently however, Rehermann et al. [1996] have been unable to find a linear correlation between peripheral blood derived cytotoxic T-lymphocyte response index or precursor frequency, and plasma HCV RNA levels. Thus, the precise contribution of the immune system to controlling viral replication during chronic HCV infection is still not completely understood. It could be speculated that the increased level of HCV replication observed in co-infected patients is in part due to factors induced by HIV infection which are generated independently of the degree of immunesupression. This is particularly relevant considering that HCV has been suggested to infect and replicate in peripheral blood mononuclear cells of hemophilic patients [Henin et al., 1994; Willems et al., 1994]. Finally, it should be noted that the elevated levels of HCV RNA detected in HIV co-infected hemophiliacs may have important implications in the transmissibility of HCV infection to their sexual partners and household members [Eyster et al., 1991].

In conclusion, we have determined that the HCV genotype distribution among Argentinean hemophilic patients is characterized by a higher proportion of type 1 infections. Although HCV viral loads were higher in HIV co-infected patients than in HIV seronegative patients, there was no correlation between HIV viral load, degree of immune suppression, and HCV viral levels. Our study did not attempt to find a correlation between infecting HCV genotype or pre-existing viral load and severity of disease assessed by liver histology or treatment outcome. These studies are currently under way.

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